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In vivo and in vitro influence of etretinate on erythrocyte membrane fluidity

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Abstract

The molecular mechanisms underlying the action of synthetic retinoids have been studied intensively, but they are not fully understood yet. It is well known that retinoids exert their effects on gene expression via the retinoic acid receptor. Some observations suggest that the main aromatic retinoid etretinate (Tigason) exerts its therapeutic effect in psoriasis also through an action on the cell membrane. In this paper, we present the results of previously unreleased experiments (when Tigason was still in use) concerning the in vivo and in vitro influence of etretinate on erythrocyte membrane fluidity in psoriatic patients. Erythrocytes from healthy subjects and topically treated psoriatics were chosen as control groups. Membrane fluidity was measured by the electron paramagnetic resonance (EPR) spin-labelling technique. Erythrocytes from psoriatic patients had lower membrane fluidity, a lower antioxidant activity and a greater susceptibility to peroxidation than those from healthy subjects. After treatment with etretinate, a significant increase in erythrocyte membrane fluidity and in antioxidant activity as well as a decrease in lipid peroxidation were observed in erythrocytes from patients. Local therapy of psoriatic lesions had no influence on the improvement in membrane fluidity and antioxidant activity of erythrocytes. Incubation of erythrocytes from healthy controls and topically treated psoriatics with etretinate in vitro confirmed its fluidizing effect on erythrocyte membranes. These data may indicate that two mechanisms lead to an increase in erythrocyte membrane fluidity in psoriatic patients treated with Tigason: the first one, indirect, by improvement of the antioxidant defence system and cell protection against lipid peroxidation, and the second one, by a direct fluidizing effect of etretinate on the erythrocyte membrane. © 2001 Published by Elsevier Science B.V.

Keywords: Psoriasis; Etretinate; Aromatic retinoid; Erythrocyte membrane fluidity; Electron paramagnetic resonance (EPR); Spin labelling

1. Introduction

Retinoids are vitamin A analogues characterised by particularly strong therapeutic activity. Oral application of retinoids has revolutionised the treatment of severe forms of psoriasis and acne and has also proved to be effective against other skin diseases. Retinoids are supposed to be first effective substances in the treatment of almost all keratinization defects (Traupe, 1989).

Tigason (Ro 10-9359) was the first aromatic retinoid introduced into dermatological practice. Its active substance is etretinate (ethyl ester of all-*trans* retinoic acid). It is well established that etretinate, like all retinoids, exerts its effect on gene expression in the nucleus via the cellular all-*trans*-retinoic acid receptor (RAR) (Petkovich et al., 1987) and 9-*cis*-retinoid receptor (RXR) (Mangelsdorf et al., 1990), which belong to the steroid/thyroid receptor family.

Etretinate has been found to inhibit inflammation, proliferation and terminal differentiation. This drug appeared to be highly effective in psoriasis. However, the exact mechanism of its action in psoriasis is unknown. Although Tigason is not used in clinical practice any more, and it has been replaced by its metabolite acitretin (Neotigason), its therapeutic efficacy is undeniable.

It has been shown that all-*trans* retinoic acid and its derivative etretinate can influence the cell membrane, modifying the metabolism of red cell membrane proteins (Kumar et al., 1983), membrane transport (Mozzato et al., 1989) and its fluidity (Varani et al., 1996; Wassall and Stillwell, 1990; Simonetti and Messini, 1989).

It is not quite clear at which stage of the disease process etretinate and other aromatic retinoids exert their strongest therapeutic effect. Therefore, investigations of the action of retinoids on plasma membranes in psoriasis may provide new data concerning the pathogenesis of the disease at the molecular level.

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A large number of observations indicate that psoriasis is linked to plasma membrane alterations in different types of cells (DiCicco et al., 1987; Ferretti et al., 1989; Offidani et al., 1989). Some authors suggest that psoriasis might be a widespread membrane disorder (Mahrle and Orfanos, 1977; Mozzato et al., 1989; Kumar et al., 1983). Therefore, it is possible that etretinate exerts its therapeutic effect in psoriasis also through an action on the cell membrane. Simonetti and Messini (1989) have shown that etretinate applied to psoriatic patients even in low doses (0.3 mg/kg by weight) caused a significant increase in erythrocyte membrane fluidity as compared with values before treatment. They also found slight (but not statistically significant) changes in phospholipid fatty acid composition and cholesterol-to-protein ratio. However, it seems that these changes in lipid composition of the bilayer cannot explain completely the observed changes in membrane fluidity. Therefore, the aim of our study was to find the cause of the increase in erythrocyte membrane fluidity in psoriatic patients treated orally with Tigason.

Since membrane fluidity is affected by different factors (Hidaka et al., 1986; Kimelberg, 1975), some of them were analysed: the cholesterol-to-phospholipid molar ratio, the activity of antioxidant enzymes (superoxide dismutase, catalase) in erythrocytes and the susceptibility to lipid peroxidation, estimated as thiobarbituric acid reactive substance products.

2. Subjects and methods

2.1. Subjects

The study comprised 25 patients with common psoriasis (11 males, 14 females, aged 24–64, mean 49). Fifteen patients (seven males, eight females, aged 24–53, mean 41) underwent conventional psoriasis therapy—local treatment with salicylic acid and tar ointment (group A). For this group, duration of treatment was between 4 and 6 weeks. Ten patients (four males, six females, aged 31–64, mean 52) were treated with etretinate (group B). The daily dose of etretinate (Tigason) was 0.8–1 mg/kg by weight. These patients were treated for 4–7 weeks. All patients were evaluated on the Psoriasis Area and Severity Index (PASI) rating scale before and after the therapy. Patients suffered from moderate psoriasis (PASI: 13.5–25.6) with a mean duration of 19 years and the mean latest recurrence 9 months. The controls were 10 healthy volunteers (four males, six females, aged 27–58, mean 51.5) without any family history of psoriasis and not on regular medication. The clinical characteristics of the healthy subjects and patients and their plasma lipid profiles are shown in Table 1. The studied subjects were not smokers and had no other diseases (i.e. diabetes mellitus, liver, kidney, cardiovascular or joint disease). Alcoholics were excluded from the study. Diet was not restricted and the subjects were instructed to adhere to their diet and not to change their drinking habits. No patient received any drug, other than etretinate, that could influence membrane fluidity (Goldstein, 1984; Abel et al., 1986; Streinkraus et al., 1991; Wolf et al., 1990; Navaratnam and Gebauer, 1990). Written informed consent was obtained from each subject and the study was approved by the regional Ethics Committee.

2.2. Isolation of erythrocytes

Blood was obtained from healthy volunteers by vein puncture and mixed with 1/10 volume of 0.13 M trisodium citrate. Erythrocytes were isolated from fresh blood by centrifugation at 4 °C, at $1500 \times g$ and purified by three cycles of resuspension and washing with phosphate-buffered saline (PBS) (310 mOsm, pH 7.4) after careful removal of the buffy coat.

Table 1
Physical and clinical characteristics of healthy subjects as well as topically and etretinate-treated psoriatic patients

	Controls	Psoriatics patients treated		ANOVA (P)
		Topically	With etretinate	
Age (years)	51.5 ± 7.0	41.0 ± 8.5[a]	52.0 ± 7.0[b]	$P < 0.001$
Duration of psoriasis (years)	–	11.0 ± 6.0	13.0 ± 7.0	NS
Broca index (kg/cm)	0.89 ± 0.12	0.91 ± 0.30	0.93 ± 0.21	NS
PASI before treatment	–	18.6 ± 4.5	20.3 ± 5.2	NS
PASI after treatment	–	7.2 ± 3.9	8.1 ± 5.0	NS
Total cholesterol (mmol/l)	5.08 ± 0.71	4.88 ± 0.59	5.17 ± 0.72	NS
Triglycerides (mmol/l)	1.24 ± 0.48	1.27 ± 0.45	1.38 ± 0.45	NS
HDL cholesterol (mmol/l)	1.16 ± 0.33	1.13 ± 0.29	1.18 ± 0.33	NS
LDL cholesterol (mmol/l)	3.68 ± 0.89	3.50 ± 0.75	3.71 ± 0.83	NS

Results are expressed as means ± standard deviation (S.D.). NS: not significant; HDL: high-density lipoproteins; LDL: low-density lipoproteins; ANOVA: analysis of variance.

[a] $P < 0.01$ versus healthy subjects.

[b] $P < 0.005$ versus topically treated patients (group A).

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2.3. Laboratory methods

Plasma cholesterol and triglycerides were measured enzymatically (Alpha Diagnostic, Poland). High-density lipoprotein cholesterol was measured by the polyethylene glycol precipitation method (Demacker et al., 1980). Low-density lipoprotein cholesterol was estimated using the Friedewald equation (Friedewald et al., 1972). Superoxide dismutase activity in erythrocytes was determined by the adrenaline method of Misra and Fridovich (1972) and re-calculated as units per gram of haemoglobin (U/g Hb). Catalase activity was estimated using the manganometric method and expressed in U/g Hb (Szczeklik, 1974). The malondialdehyde level was assayed according to the method of Placer et al. (1966). Lipids were extracted from erythrocyte membranes by chloroform/methanol (2:1, v/v) followed by chloroform/methanol/water (60:30:20, v/v/v) according to Alling et al. (1984). Cholesterol and phospholipid contents were assayed colorimetrically: the first one by the Higgins (1988) and the second one according to method of Stewart (1980).

2.4. Spin labels

Fatty acid spin labels (5-doxylstearic acid and 12-doxylstearic acid) were purchased from Sigma (St. Louis, MO, USA). For each spin label stock solutions (0.1 mg/ml in ethanol) were prepared and kept at −80 °C.

2.5. EPR spin label measurements

Labelling was performed on a thin film of the spin probe formed by evaporation of 150 μl of stock solution under a flow of nitrogen gas in an *Eppendorf* tube (Ogura et al., 1988).

Erythrocytes, 50 μl, were put into a test tube containing a thin film of the label and incubated at 37 °C for 15 min. After being washed twice with 5 ml of PBS, the labelled erythrocytes were put into a quartz capillary tube and placed in the cavity of EPR Radiopan spectrometer. EPR spectra were obtained at X-band (9.4 GHz), at modulation frquency of 100 kHz. The scan time was 4 min, and the time constant was 0.3 s. All spectra were recorded at room temperature.

Generally, the fluidity of the membrane can be estimated from the order parameter S, which can vary between 0 and 1. Changes in the order parameter values correspond to changes in membrane local viscosity—an increase means a decrease in viscosity. The S parameter was calculated from the formula of Gaffney (1976):

$$S = \frac{A_{\parallel} - (A_{\perp} + C)}{A_{\parallel} + 2(A_{\perp} + C)} \times 1.723 \qquad (1)$$

$$C = 1.4 - 0.053(A_{\parallel} - A_{\perp})$$

where $2A_{\parallel}$ and $2A_{\perp}$ are parallel and perpendicular hyperfine splitting parameters of the spectrum, respectively.

2.6. In vitro interaction of etretinate with erythrocyte membrane

Etretinate, kindly provided by Hoffmann-La Roche (Basel), was dissolved in dimethyl sulfoxide (DMSO) and appropriate amounts were added to the 1-ml suspensions of labelled erythrocytes (5×10^6 cells/ml in PBS containing 0.1% glucose) from healthy controls and patients. The final concentration of etretinate was 1.4×10^{-9} M. This concentration was chosen to provide an etretinate/red cell ratio nearly similar to that obtained in plasma during long-term Tigason therapy (50 mg/day). The concentra-

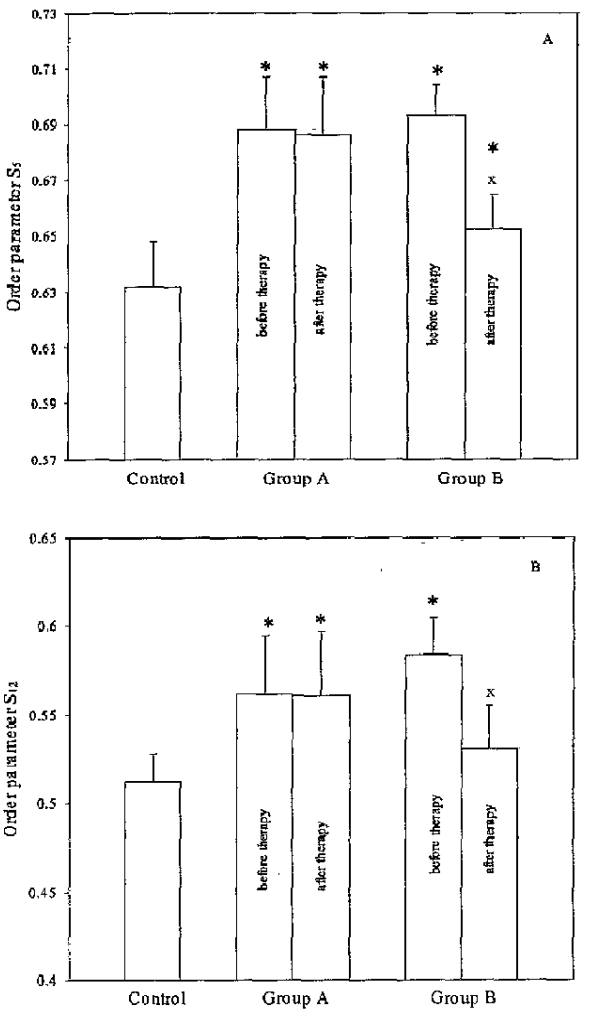

Fig. 1. Mean values ± S.D. of the order parameters S_5 (A) and S_{12} (B) in erythrocytes from healthy controls and psoriatic patients (groups A and B) before and after therapy; (*) significantly different from healthy control, $^{*}P < 0.05$; (×) significantly different from values before treatment, $^{\times}P < 0.05$.

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tion of DMSO in erythrocyte suspensions never exceeded 0.1%. This concentration did not affect the membrane fluidity of erythrocytes. Erythrocyte suspensions were incubated for 30 min at 37 °C. After removal of the media, the cells were washed twice with PBS and 50 μl of erythrocytes packed in capillary tube was placed in the cavity of the EPR spectrometer and spectra were recorded at room temperature.

2.7. Statistical analysis

All results are presented as means ± S.D. The distribution of variables was tested for normality using the Shapiro–Wilk test. Differences between specific means were tested by one-way analysis of variance (ANOVA) with post hoc analysis using Scheffe's test. The non-parametric paired Wilcoxon test was used to compare order parameter values before and after incubation with etretinate. The unpaired Mann–Whitney test was used to compare the studied groups. A value of $P < 0.05$ was accepted as statistically significant.

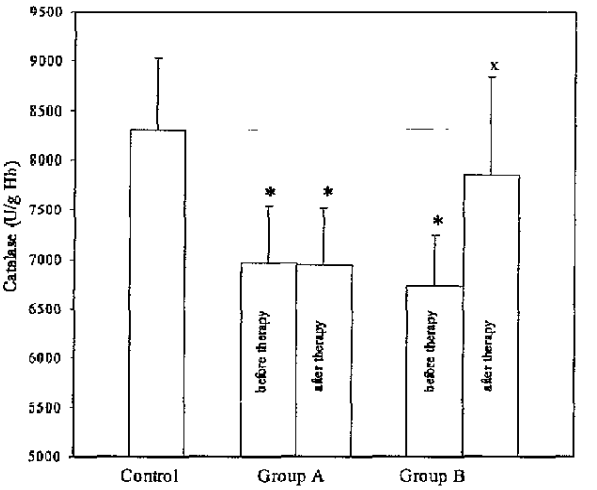

Fig. 3. Mean values ± S.D. of catalase activity in erythrocytes from healthy controls and psoriatic patients (groups A and B) before and after therapy; (*) significantly different from healthy control, $^{*}P < 0.05$; (×) significantly different from values before treatment, $^{\times}P < 0.05$.

3. Results

The physical and clinical characteristics of the study groups are summarized in Table 1. The healthy subjects and two psoriatic groups differed with respect to age, but did not differ with respect to Broca index and plasma lipid parameters. There were no significant PASI score differences between the two psoriatic groups (groups A and B) before and after therapy. The erythrocyte membrane lipid composition in controls and patients did not differ and in both psoriatic groups did not reveal significant changes in the cholesterol-to-phospholipid molar ratio before and after therapy (data not shown).

The basal values of the order parameters for the studied groups differed (Fig. 1A and B). The order parameter values S_5 and S_{12} of erythrocyte membranes from psoriatic patients were higher (lower membrane fluidity) than those recorded for the membranes of healthy controls. In topically treated patients (group A), both S_5 and S_{12} order parameters after therapy were only slightly decreased. This

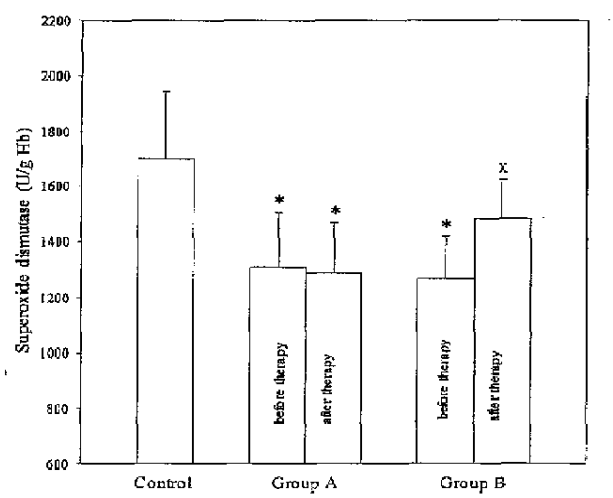

Fig. 2. Mean values ± S.D. of superoxide dismutase activity in erythrocytes from healthy controls and psoriatic patients (groups A and B) before and after therapy; (*) significantly different from healthy control, $^{*}P < 0.05$; (×) significantly different from values before treatment, $^{\times}P < 0.05$.

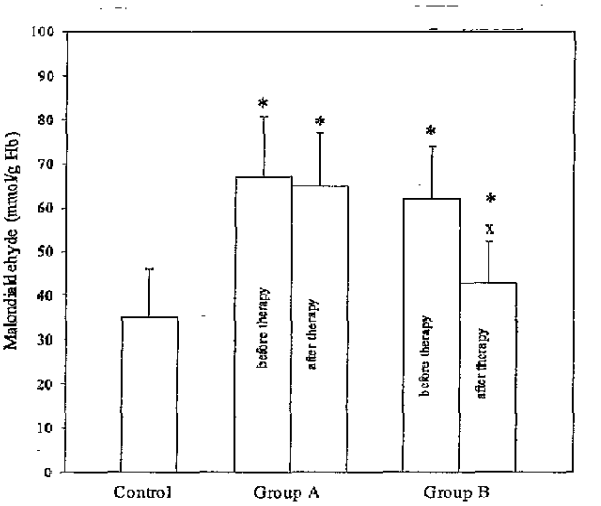

Fig. 4. Mean values ± S.D. of malondialdehyde in erythrocytes from healthy controls and psoriatic patients (groups A and B) before and after therapy; (*) significantly different from healthy control, $^{*}P < 0.05$; (×) significantly different from values before treatment, $^{\times}P < 0.05$.

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change was, however, statistically insignificant. It indicates that local therapy for psoriasis has no influence on the change in erythrocyte membrane fluidity. In patients treated orally with Tigason (group B), a significant decrease in the order parameter values (S_5, S_{12}) appeared, but after therapy, they were still higher than in the control group (Fig. 1A and B). Therefore, it may be presumed that in patients from group B, in which clearing of lesions was observed (decrease of PASI), the order parameters tended to decrease, which means that membrane fluidity increased. Tigason treatment exerted a more evident fluidizing effect in the deeper hydrocarbon domains of the bilayer (decrease of S_{12} by about 9%, $P < 0.001$) than in the polar head region (decrease of S_5 by about 6%, $P < 0.001$).

The basal values of catalase and superoxide dismutase in erythrocytes from the different groups differed (Figs. 2 and 3). The activity of superoxide dismutase and of catalase in erythrocytes from psoriatics was significantly decreased as compared with that for erythrocytes from healthy controls. In group B, a significant increase in catalase and superoxide dismutase was observed after therapy. Thus, during Tigason treatment, the activity of both erythrocyte enzymes systematically increased, but in the phase of clinical remission, enzyme activity had not quite normalized. In patients from group A, the activity of the examined enzymes after therapy was not statistically significantly changed. It indicates that topical therapy of psoriasis has no influence on the improvement of erythrocyte antioxidant activity.

The levels of malondialdehyde in patients before treatment were significantly higher than those in the control group (Fig. 4). After therapy in Tigason-treated patients (group B), there was a significant decrease in malondialdehyde levels. In topically treated patients (group A), malondialdehyde levels after therapy were not statistically significantly changed as compared with values before treatment.

The in vitro influence of etretinate on membrane fluidity depended on the source of the erythrocytes (Fig. 5A and B). In erythrocytes from healthy controls and topically treated psoriatics, etretinate induced a marked elevation of membrane fluidity. However, it did not change significantly in vitro the membrane fluidity of erythrocytes from Tigason-treated patients.

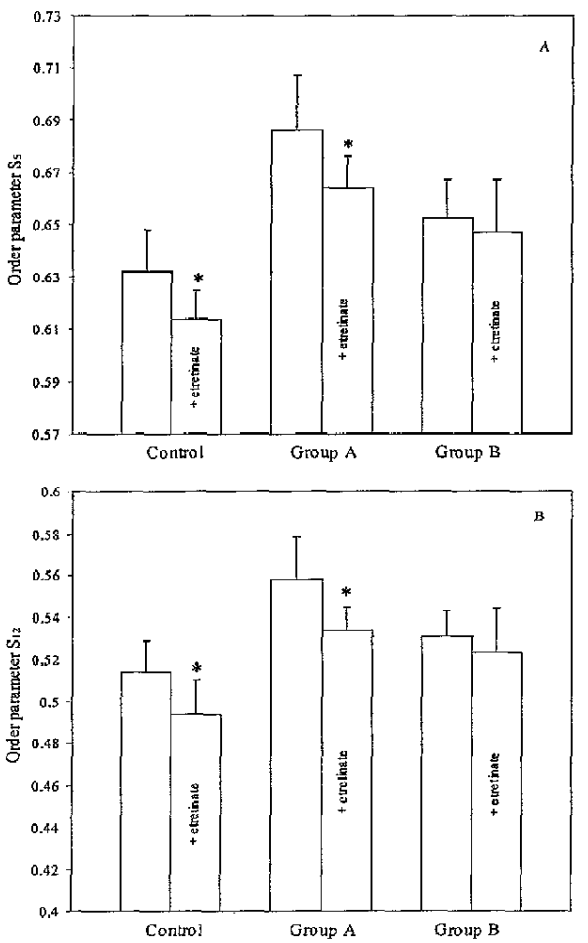

Fig. 5. Effect of in vitro incubation with etretinate on the order parameters S_5 (A) and S_{12} (B) in erythrocyte membranes from healthy controls and psoriatic patients (groups A and B). Significantly different from control erythrocytes incubated in medium only, $^*P < 0.05$.

4. Discussion

The present study investigated the in vivo and in vitro interaction of etretinate with erythrocyte membranes by analysing lipid fluidity with the use of the EPR spin probe technique. Erythrocyte antioxidant enzymes, susceptibility to lipid peroxidation, membrane cholesterol-to-phospholipid ratio and plasma lipid parameters in healthy volunteers and patients were examined because these are the principal determinants of membrane fluidity.

The erythrocyte membrane is a sensitive model to reveal complex alterations of lipid metabolism. Actually, mature erythrocytes lack lipid biosynthetic pathways and therefore, their membrane stability depends on exchange processes with plasma lipoproteins. Therefore, we selected patients and healthy subjects with a similar plasma lipid profile, in order to exclude the influence of abnormalities of plasma lipid metabolism on membrane lipid composition. Moreover, we excluded the possibility of differences in fluidity arising due to obesity, by selecting patients and controls with a similar Broca's index. However, the studied subjects differed with respect to age. However, as has been shown by Butterfield et al. (1982), such differences in age (mean 10 years) do not influence membrane fluidity changes.

Our data show that the basal fluidity of erythrocyte membranes from healthy subjects and patients is different, but this difference does not seem to be linked to membrane

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lipid composition or plasma lipid profile. These results are in agreement with those reported by other authors (Ferretti et al., 1989; Offidani et al., 1989). The data showed that the membrane fluidity of erythrocytes was decreased more evidently in the region of the bilayer sampled by 12-doxyl stearate than in the polar head region sampled by 12-doxyl stearate. This result could be explained on the basis of the different effect of lipid peroxidation on membrane fluidity. As was shown by Bruch and Thayer (1983), the effect of lipid peroxidation on fluidity is maximal in the membrane microenvironment sampled by 12-doxyl stearate. This is because the region of the bilayer is expected to contain a large proportion of fatty acid double bonds susceptible to lipid peroxidation.

This study demonstrated that in psoriatic patients treated orally with Tigason (0.8–1.0 mg/kg by weight), the order parameters decreased (increase in membrane fluidity) as treatment progressed. Our findings are consistent with the observations of Simonetti and Messini (1989). They showed that etretinate applied in psoriatic patients even in low doses (0.3 mg/kg by weight) caused a significant increase in erythrocyte membrane fluidity as compared with values before treatment. We observed that Tigason treatment had a more evident fluidizing effect in the deeper hydrocarbon domains of the bilayer than in the polar head region. Based on results obtained by Bruch and Thayer (1983) (cited above), it may be supposed that the antioxidant action of etretinate accounts for this effect.

Although membrane fluidity is influenced by the membrane phospholipid composition (Cooper, 1977; Hidaka et al., 1986; Shinitzky and Inbar, 1976), there was no alteration in the membrane lipid composition of erythrocytes from patients treated with Tigason in this study. Simonetti and Messini (1989) found slight (but not statistically significant) changes in phospholipid fatty acid composition and cholesterol-to-protein ratio during etretinate therapy. This discrepancy may result from the fact that etretinate may inhibit the enzymatic deacylation of arachidonic acid from membrane phospholipids via phospholipase A_2 (Nigam, 1987). However, the activation of phospholipase A_2, which is located in the membrane, brings about changes in the membrane lipid composition.

Our experiments indicate that the modification of membrane fluidity caused by etretinate results from an increase in erythrocyte antioxidant enzyme activity. In patients treated orally with Tigason, a significant increase in superoxide dismutase and catalase activities in erythrocytes was observed after the end of therapy. These results were not observed in the group of patients treated only topically, in spite of the regression of skin lesions (decrease in PASI). In these patients after the end of therapy, the order parameter was unchanged or had only changed slightly, and superoxide dismutase and catalase activities were not significantly higher than the initial values.

Kaszuba et al. (1996) have also found that the activity of superoxide dismutase in erythrocytes was significantly higher at the end of combined therapy (Retinoids + Psoralen-Ultraviolet A + Cignoline) as compared with values before treatment. Thus, the therapeutic effect of etretinate also results from its influence on the production of reactive oxygen species (ROS). In fact, it has been reported that retinoids have an inhibitory effect on the generation of superoxide (O_2^-) by stimulated PMNs as well as on the migration and accumulation of these cells (Camisa et al., 1982; Witz et al., 1980). Other authors have observed that retinoids exert an inhibitory effect also on other ROS, including hydroxyl radicals ($OH^\cdot$) and hydrogen peroxide (H_2O_2) (Yoshioka et al., 1986; Hiramatsu and Packer, 1990; Bohne et al., 1997).

It is well known that peroxidation induces modifications of the physicochemical state of membranes and results, among others, in changes in membrane fluidity. The data for malondialdehyde levels, which indicate an increase in the oxidation of polyunsaturated fatty acids, could confirm the hypothesis that the decrease in membrane fluidity in psoriatic patients is due to enhanced lipid peroxidation within the bilayer during the active phase of psoriasis (Górnicki, 1999). Our results also indicate that Tigason therapy improves the antioxidant defence system and protects cells against lipid peroxidation. These effects may increase erythrocyte membrane fluidity in Tigason-treated patients.

To find out whether Tigason exerts its effect on membrane fluidity only by improving the antioxidant potential of the cell or by affecting the cell membrane, the in vitro interaction of etretinate with erythrocyte membranes was examined. The in vitro influence of etretinate on membrane fluidity depended on the source of the erythrocytes. In the erythrocytes of healthy controls and topically treated psoriatics, etretinate induced an elevation of membrane fluidity. However, in vitro, it did not change significantly the membrane fluidity of erythrocytes from Tigason-treated patients. This is probably because in these patients, etretinate moieties had already caused a fluidizing effect on the erythrocyte membrane. These data show that the washing procedure during preparation of red cells before labelling was not able to wash out entirely etretinate moieties incorporated within the membrane bilayer. The results show that also in vitro, etretinate exerts a fluidizing effect on the erythrocyte membrane. These data indicate that probably two mechanisms lead to an increase in erythrocyte membrane fluidity in psoriatic patients treated with Tigason: the first one, indirect, by improvement of the antioxidant defence system and cell protection against lipid peroxidation, and the second one, by a direct fluidizing effect of etretinate on the erythrocyte membrane.

Since nuclear receptors for 9-*cis*-retinoic acid in different types of cells (keratinocytes, melanocytes, fibroblasts, leukocytes) have been discovered, the prevailing concept is that the biological action of retinoids occurs only through binding with receptors and modulation of the expression of genes responsible for cell growth and differentiation

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(Mouchon et al., 1999; Feng et al., 1997). The results presented in this paper indicate that an equally essential mechanism of therapeutic action of etretinate is its influence on cell membranes.

Thus, the mechanism of the therapeutic action of etretinate in psoriasis is complex and it is not entirely recognised. In our opinion, further investigations should be performed.

Since Tigason is no longer used in clinical practice, it would be worth carrying out similar in vivo and some in vitro experiments using its metabolite acitretin (Neotigason) because clinical experiments have revealed that acitretin can be transformed into etretinate under some conditions.

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